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Supplemental Information

Modulation of Asymmetric Division Diversity through Cytokinin and SPEECHLESS Regulatory Interactions in the *Arabidopsis* Stomatal Lineage

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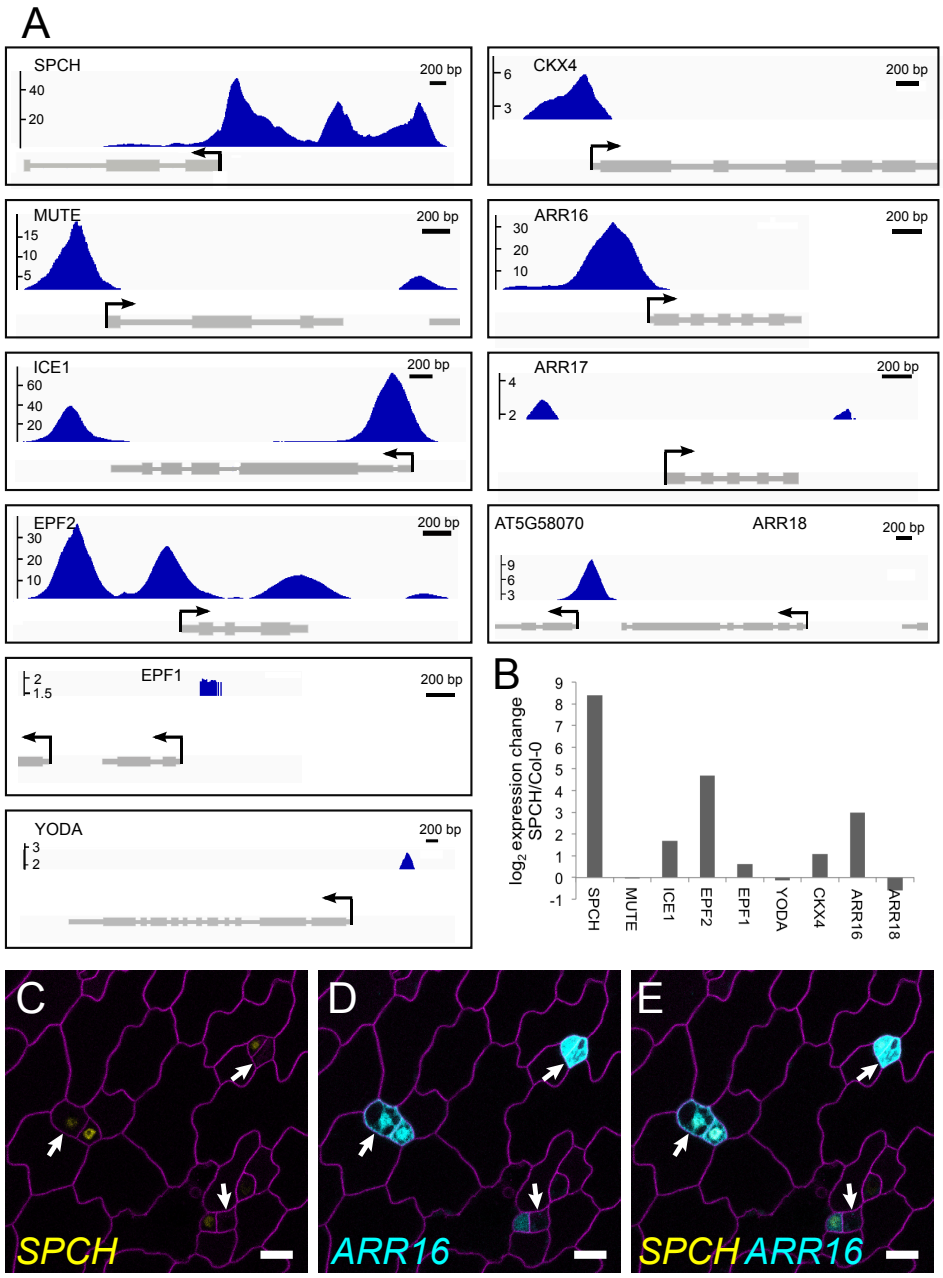

Figure S1: Identification of CK pathway genes as stomatal lineage or SPCH targets, related to Figure 1.

A) SPCH ChIP-seq profiles of the key stomatal regulators and set of CK metabolism and signaling associated genes (from data in Lau et al., 2014). ChIP-seq was performed on 4 day old Col-0 and *pSPCH-SPCH2-4A-MYC*;*spch* using antibodies against the MYC epitope. Peaks scores of higher than 3 are considered significant (FDR $<10^{-6}$). Arrows indicate transcriptional start sites and orientation of genes.
B) Response of genes in (A) to 8-hr estradiol induction of *pEST-SPCH-1-4A* vs. control derived from RNA-seq on 4 day old seedlings (from data in Lau et al., 2014). This timepoint was chosen because MUTE, a marker of GMC identity is not yet induced, and we wished to avoid changes in cell identity that might have secondary effects on gene expression. Y-axis values represents $\log_2$-transformed expression values.
C–E) Coexpression of SPCH and ARR16 in the epidermis of cotyledons in 3 day old seedlings. Arrows point to persistence of ARR16 in SLGCs (SLGCs are distinguished by relatively low SPCH compared to smaller neighbor cell) after an asymmetric division C) *pSPCH-SPCH-YFP*, D) *pARR16-ARR16-CFP* and E) merged. Cell outlines (magenta) marked with *pML1pro-mCherry-RCI2A*; scale bars, 10 μm.

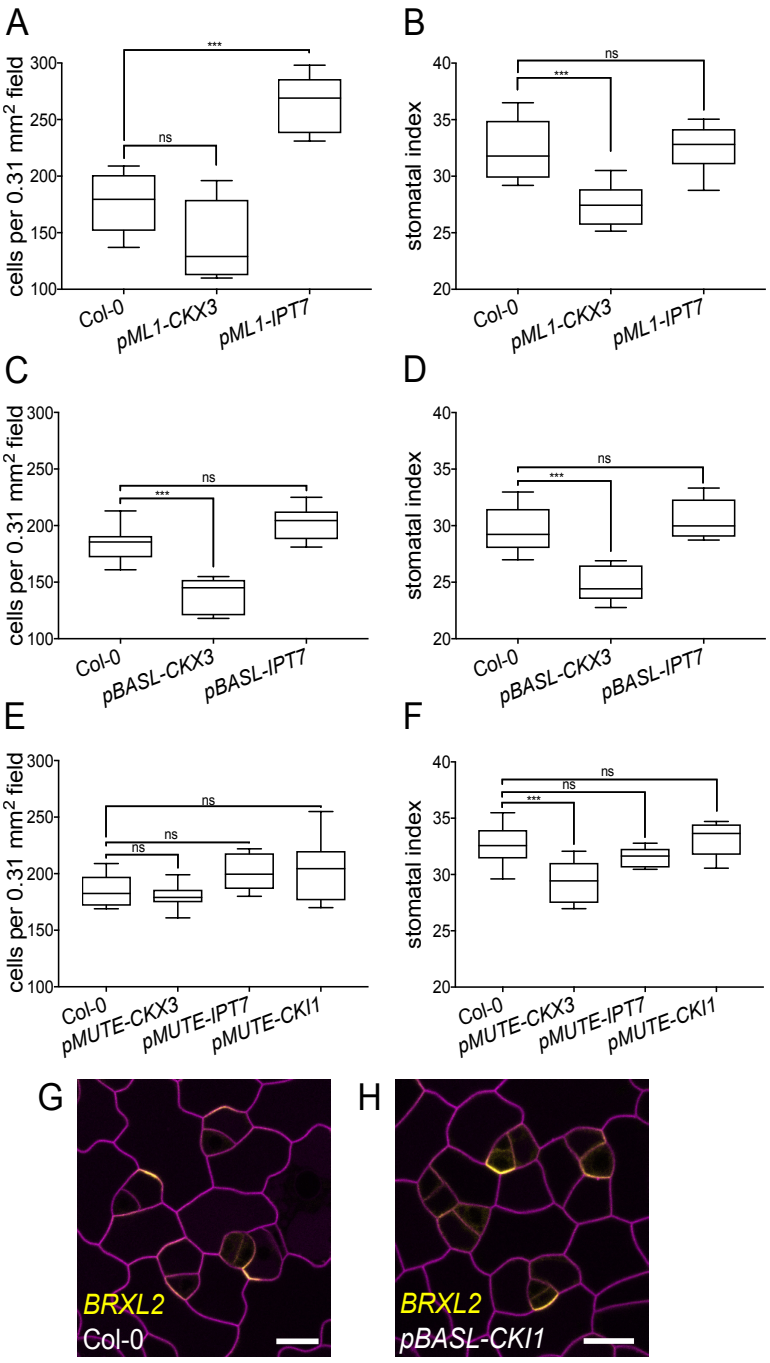

Figure S2: Transgenic manipulation of CK levels changes cell division competence and SI but does not alter cell polarity, related to Figure 2.

Quantification of epidermal cell numbers and SI in 10 day old cotyledons, from same genotypes analyzed at day 4 in Figure 2.
A–B) Col-0, *pML1-CKX3,* and *pML1-IPT7* (n=8/genotype).
C–D) Col-0, *pBASL-CKX3*, and *pBASL-IPT7* (n=8/genotype). Cell numbers in 10 day old *pBASL-CKI1* lines could not be accurately quantified because excessive divisions lead to a distorted epidermal surface.
E–F) Col-0, *pMUTE-CKX3*, *pMUTE-IPT7*, and *pMUTE-CKI1* (n=8 genotype).
G) Polarity marker *BRXL2-BRXL2-YFP* expression in 7 day old Col-0 and (H) *pBASL-CKI1* true leaves. Scale bars, 10 μm. ***p<0.001 by Dunnet's test.

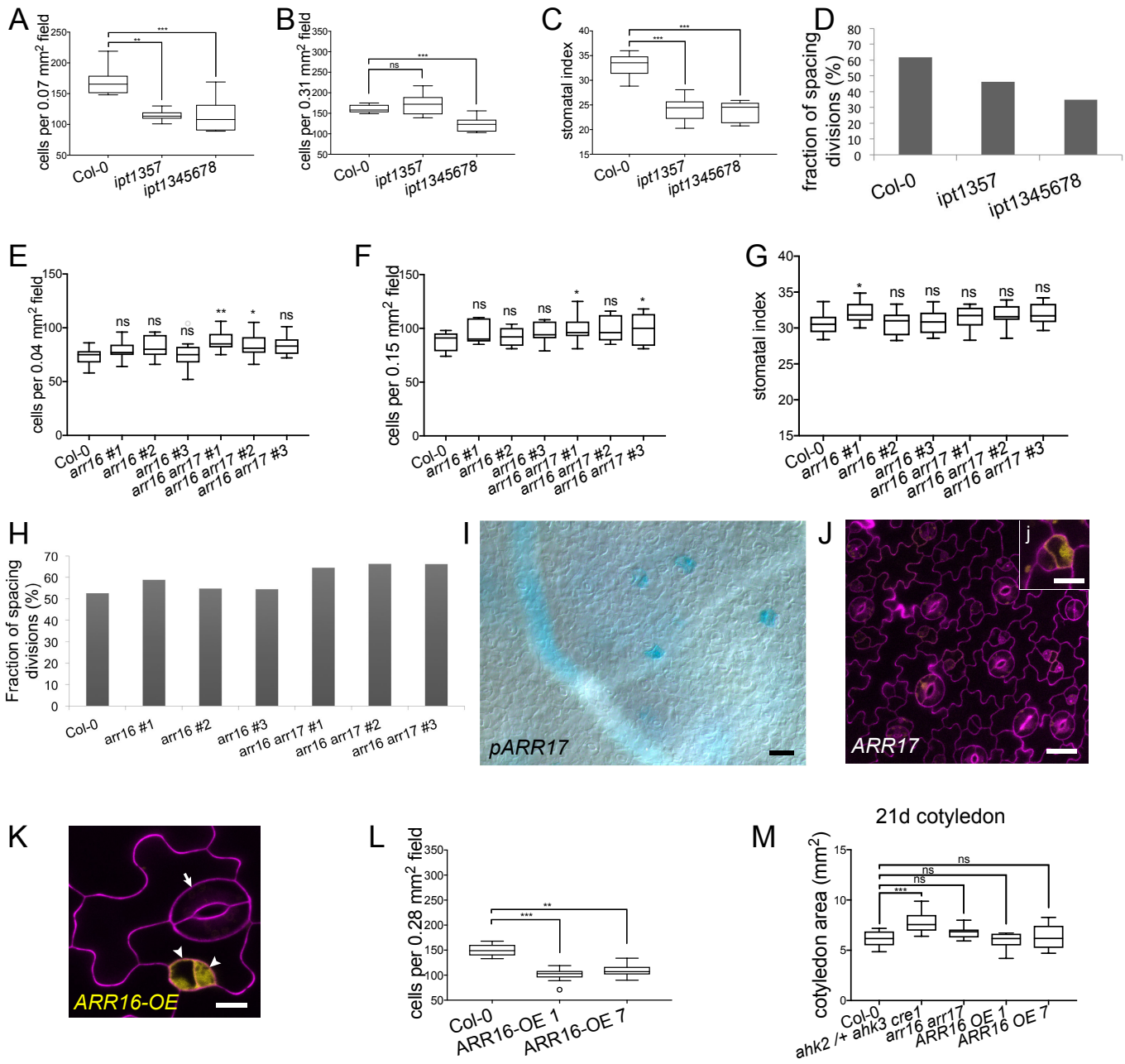

Figure S3: Members of CK signaling pathway modulate cell division potential in SLGCs, related to Figure 3.

A–C) Quantification of cotyledon epidermis phenotypes in Col-0 and CK biosynthesis mutants *ipt1357* and *ipt1345678* (n>10/genotype), (A) cell number at 4 days (B) cell number at 10 days and (C) SI at 10 days.
D) Fraction of SLGCs going through spacing divisions in 4 day old Col-0 (n=306), *ipt1357* (n=323), and *ipt1345678* (n=379) on the adaxial side of the cotyledons. Co-0 quantification from Figure 3D was used as a control. At least 13 cotyledons per genotype were scored.
E-G) Quantification of cotyledon epidermis phenotypes in Col-0, and three independent lines of *arr16* and *arr16 arr17* (n=11/genotype), (E) cell number at 4 days (F) cell number at 10 days and (G) SI at 10 days.
H) Fraction of SLGCs going through spacing divisions in 4 day old Col-0 (n=574), *arr16 #1* (n=413), *arr16 #2* (n=816), *arr16 #3* (n=591), *arr16 arr17 #1* (n=511), *arr16 arr17 #2* (n=715), and *arr16 arr17 #3* (n=654) on the adaxial side of the cotyledons. At least 12 cotyledons per genotype were scored.
I) Brightfield image of *pARR17-GUS* (blue), expressed sporadically in the leaf epidermis and in the inner part of the developing leaf in 7 day old seedling.
J) Expression of translational *pARR17-gARR17-YFP* reporter is observed sporadically in the mature GCs and (j) SLGCs in true leaves of 10 day old seedling. J and j are from different seedlings.
K) Confocal image of 4 day old cotyledon overexpressing (OE) *ARR16-YFP* with the TMM promoter to show early stomatal lineage focused expression. Arrowhead, early stomatal lineage cell with high *ARR16* expression levels; arrow, mature stomata without *ARR16* expression. Cell outlines were marked with PI. Scale bar, 10 μm.
L) Quantification of cell number in Col-0 and two independent ARR16-OE lines (n=11/genotype) at 10 day old cotyledon epidermis.
M) Quantification of cotyledon area in Col-0, *ahk2/+ ahk3 cre1*, *arr16 arr17* #1 and two independent ARR16-OE lines (n=9-12/genotype) at day 21.
*p<0.05, **p<0.01, ***p<0.001 by Dunnett's test (B-C, E-G, M) or by Dunn's test (A, L).

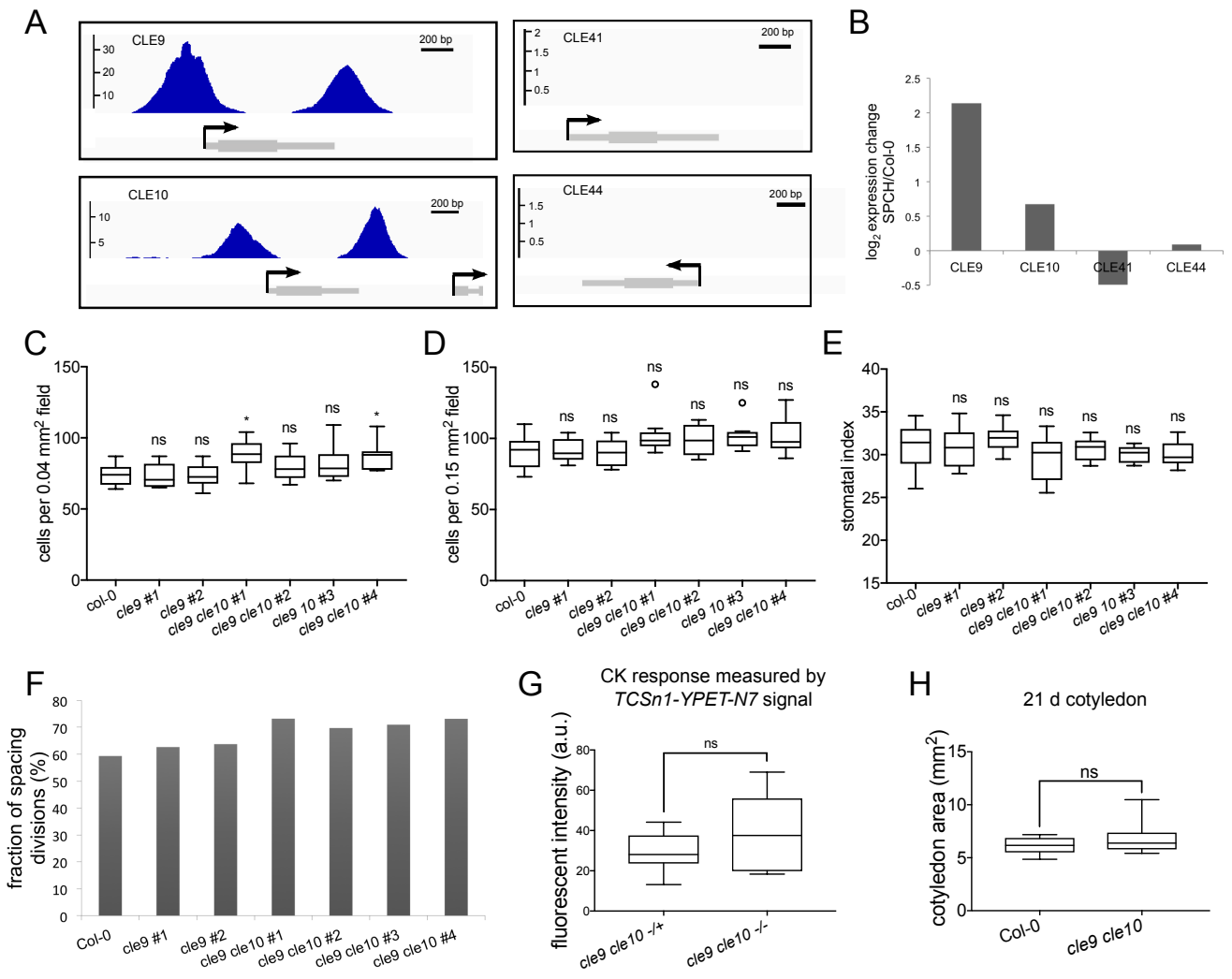

Figure S4: Evidence that CLE9 and CLE10 are SPCH targets and analysis of additional CRISPR alleles, related to Figure 4.

A) SPCH ChIP-seq profiles of the genes encoding CLE peptides (from data in Lau et al., 2014). ChIP-seq was performed on 4 day old Col-0 and *pSPCH-SPCH2-4A-MYC*;*spch* using antibodies against the MYC epitope. Peaks scores of higher than 3 are considered significant (FDR <10^{-6}). Arrows indicate transcriptional start sites and orientation of genes.
B) Response of genes in (B) to 8-hr estradiol induction of *pEST-SPCH-1-4A* vs. control derived from RNA-seq on 4 day old seedlings (from data in Lau et al., 2014). Y-axis values represents $\log_2$-transformed expression values.
C-E) Quantification of cotyledon epidermis phenotypes in Col-0, and two independent *cle9* and four independent *cle9 cle10* lines (n=10/genotype), (C) cell number at 4 days (D) cell number at 10 days and (E) SI at 10 days.
F) Fraction of SLGCs going through spacing divisions in 4 day old Col-0 (n=836), *cle9 #1* (n=474), *cle9 #2* (n=433), *cle9 cle10 #1* (n=709), *cle9 cle10 #2* (n=641), *cle9 cle10 #3* (n=875), and *cle9 cle10 #4* (n=298) on the adaxial side of the cotyledons. At least 7 cotyledons per genotype were scored.
G) Quantification of fluorescence intensity of *TCSn1-2xYPET-N7* in SLGCs at abaxial side of cotyledons in 4d outcrossed heterozygous *cle9 cle10* -/+ (n=7) and backcrossed homozygous *cle9 cle10* (n=10) FI seedlings (a.u., arbitrary unit).
H) Quantification of cotyledon area in Col-0 (n=10) and *cle9 cle10 #1* (n=12) at day 21. *p<0.05, **p<0.01 by Dunnett's test (D-E), Dunn's test (C), or by Mann-Whitney test (G-H).

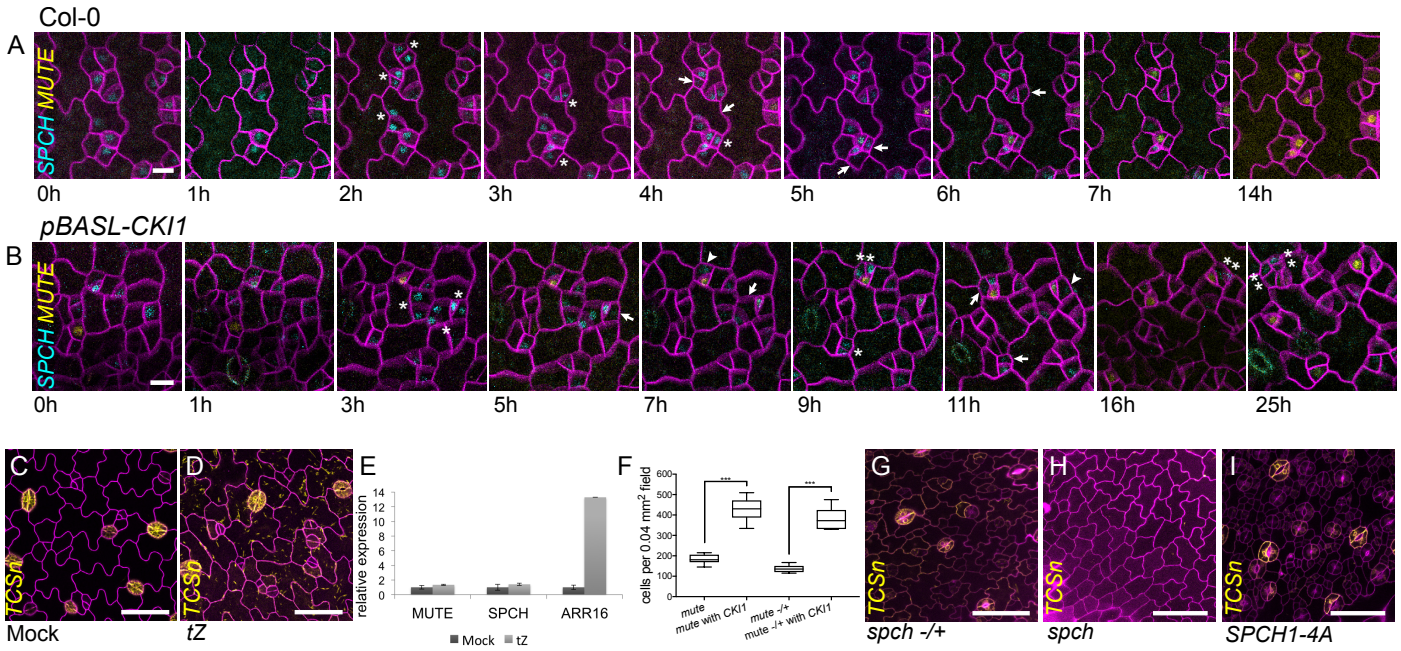

Figure S5: SPCH and CK form an expression feedback loop, related to Figure 5.

A-B) Extended timelapse of *pSPCH-SPCH-CFP; pMUTE-MUTE-YFP; pML1-mcherry-RC12A* in Col-0 (A) and *pBASL-CKI1* (B) 3 day old seedlings. Single asterisk, *SPCH* expressing cell divides; arrow, reduced *SPCH* expression in one of the daughter cells; arrowhead, increased *SPCH* levels in SLGC; two asterisks, divided SLGC. Timepoint in the movie is indicated below each image. A subset of the images presented in Figure S5-B are presented in Figure 5A-B and same labeling has been used. This excerpting means that markings are shown sometimes in later timepoints compared to Figure S5A-B. In Figure S5A two arrows indicating reduced SPCH expression are shown at 5h timepoint whereas in Figure 5A they are shown at 6h timepoint. In Figure S5B two set of double asterisks indicating SLGC division are shown at 9h and 16h timepoints whereas in Figure 5 they are shown at 11h and 25h timepoints.

C–D) *TCSn-GFP-ER* intensity is increased by 5 hour 10 μM *tZ* treatment at adaxial side of cotyledons in 3d old seedlings.

E) qRT-PCR analysis of *MUTE*, *SPCH*, and *ARR16* expression as a response to 30 minute 10μM *tZ* treatment. Expression values are normalized to the control gene *ACTIN2* and are relative to the expression in the mock treated seedlings.

F) Quantification of total cell number per 0.04 mm^2 in 4 day old *mute* and heterozygous or WT siblings transformed with *pBASL-CKI1* or a control transgene with same antibiotic resistance (n=10/genotype). Analysis was done on 4d old etiolated T1 seedlings.

G) *TCSn-GFP-ER* is expressed in the GCs and subset of SLGCs in the true leaves of 7 day old wild-type, however, in *spch* (H) *TCSn1-GFP-ER* signal is severely reduced.

I) *TCSn-GFP-ER* expression restricted to GCs and is absent from meristemoids and SLGCs in 7 day old true leaves of *pSPCH-SPCH1-4A-CFP*. Scale bars in A-B, 10 μm; scale bars in C-D, G-I 50 μm. ***p<0.001 by Student t-test.

Table S1. CRISPR induced mutations used in this study, related to Figure 3, S3, Figure 4, and S4.

Locus	Mutation	Nucleotide change	Effects on protein
ARR16	*arr16-1*	Insertion of T at nt position 12, leads to premature stop codon	Out of frame after 4 aa, 17 aa protein
ARR16	*arr16-2*	Deletion of 5 nt (TTCAG) at nt position 9, leads to premature stop codon	out of frame after 1 aa, 15 aa protein
ARR16	*arr16-3*	Insertion of A at nt position 12, leads to premature stop codon	Out of frame after 4 aa, 17 aa protein
ARR17	*arr17*	Insertion of T at nt position 99, leads to premature stop codon	Out of frame after 32 aa, 35 aa protein
CLE9	*cle9-1*	Insertion of C at nt position 74, leads to premature stop codon	Out of frame after 24 aa, 52 aa protein
CLE9	*cle9-2*	Insertion of A at nt position 74, leads to premature stop codon	Out of frame after 24 aa, 52 aa protein
CLE10	*cle10-1*	Insertion of A at nt positions 15 and 65	Out of frame after 5 aa, 118 aa protein
CLE10	*cle10-2*	Large deletion at nt position 50, leads to premature stop codon	Out of frame after 5 aa, 70 aa protein
CLE10	*cle10-3*	Large deletion at nt position 60	Out of frame after 5 aa, 107 aa protein
CLE10	*cle10-4*	Large deletion at nt position 15, leads to premature stop codon	Out of frame after 5 aa, 21 aa protein

Effects of CRISPR/Cas9 induced editing at DNA and protein level in mutant lines created for this study. nt, nucleotide; aa, amino acid.

Table S2. CRISPR induced mutant lines and their edited nucleotide sequences, related to Figure 3, S3, Figure 4, and S4.

List of all the mutant lines created by CRISPR/Cas9-induced editing for this study. The last column (nucleotide sequence) shows the WT genomic sequences and the altered target sequences in each mutant.

Line	Locus	Mutation	Nucleotide sequence
Col-0	*ARR16*	Wild-type	ATGAACAGTTCAGGAGGTTCTTGTTCGTCTTTAATGGATGTGGTGGCTTAT GATCATCATCTTCATCATGGTCATGATGAAGAGCTTCATGTTTTAGCTGTAG ATGATAATCTTATTGACCGTAAACTCGTTGAGAGGTTGCTCAAGATCTCTT GTTGCAAAGgttcaatcattttaccttcatgttttataatcaatcattactttatgcaatctttgtctctaaaatgtg aatttttgcagTGACAACAGCAGAGAATGCGCTTAGAGCATTGGAGTATTTGGGT TTGGGAGATCAAAATCAGCATATTGATGCATTGACCTGTAACgtaagcaaaaaa aaaacatataaaatcttcaagatctatggatttttttccttaggacatatttgatattaggtttatatatgtgtggattttc tagGTTATGAAGGTGAGTCTAATCATCACCGATTACTGTATGCCTGGAATGA CAGGTTTTGAGCTACTCAAGAAAGTGAAGgtaattatataacagcttcttggaaccaatatc cagagatttatattaacctaaaaatcttctgtgtgtttctttagCAGGAGTCATCAAATCTGAGAGA GGTTCCTGTTGTGATAATGTCTTCAGAGAATATTCCTACTCGCATAAACAAg taagaacaaattgagatcagattaagaacttgtttctgtgtttcttatagtgtaagtaattagtaaag
arr16 #1	*ARR16*	*arr16-1*	ATGAACAGTTC**T**AGGAGGTTC
arr16 #2	*ARR16*	*arr16-2*	ATGAACAG - - - - - GAGGTTC
arr16 #3	*ARR16*	*arr16-3*	ATGAACAGTTC**A**AGGAGGTTC
arr16 17#1	*ARR16*	*arr16-1*	ATGAACAGTTC**T**AGGAGGTTC
arr16 17#2	*ARR16*	*arr16-1*	ATGAACAGTTC**T**AGGAGGTTC
arr16 17 #3	*ARR16*	*arr16-1* *arr16-3*	ATGAACAGTTC**A**AGGAGGTTC ATGAACAGTTC**T**AGGAGGTTC
Col-0	*ARR17*	Wild-type	GTAAACT
arr17	*ARR17*	*arr17*	GTAA**T**ACT
arr16 17 #1	*ARR17*	*arr17*	GTAA**T**ACT
arr16 17 #2	*ARR17*	*arr17*	GTAA**T**ACT
arr16 17 #3	*ARR17*	*arr17*	GTAA**T**ACT
Col-0	*CLE9*	Wild-type	CTCCACC
cle9 #1	*CLE9*	*cle9-1*	CTCCA**C**CC
cle9 #2	*CLE9*	*cle9-2*	CTCCA**A**CC
cle9 10 #1	*CLE9*	*cle9-2*	CTCCA**A**CC
cle9 10 #2	*CLE9*	*cle9-2*	CTCCA**A**CC
cle9 10 #3	*CLE9*	*cle9-2*	CTCCA**A**CC
cle9 10 #4	*CLE9*	*cle9-2*	CTCCA**A**CC
Col-0	*CLE10*	Wild-type	ATGAAGACTAACCGGAACCGTCCGATCAACATCCTCATCGTCTTCTTCCTT CTTACGACCGCAAGAGCAGCAACAAGAAACTGGACCAACCGAACTCACCG AACCGTCCCTAAGGTT
cle9 10 #1	*CLE10*	*cle10-1*	ATGAAGACTAACCGG**A**AACCGTCCGATCAACATCCTCATCGTCTTCTTCCT TCTTACGACCGCAA**A**GAGCAG
cle9 10 #2	*CLE10*	*cle10-2*	ATGAAGACTAACCGGAACCGTCCGATCAACATCCTCATCGTCTTCTTCCT - - - -
cle9 10 #3	*CLE10*	*cle10-3*	ATGAAGACTAACCGGAACCGTCCGATCAACATCCTCATCGTCTTCTTCCTT CTTACGACC - - - - -
cle9 10 #4	*CLE10*	*cle10-4*	ATGAAGACTAACCGG - - - - -

Dashed line, deletion; bold, insertion; underlined, ATG.

Table S3. Type-B ARR binding motifs are frequent around the *SPCH* locus, related to Figure 5.

Analysis of the frequency of type-B ARR binding core motifs 5´-(A/G)GAT(C/T)-3´ in *SPCH* and *MUTE* loci. Previous studies defined that CK regulated type-A ARRs contain the type-B ARR binding motif on average once in every 86 base pairs (bp) whereas non-CK regulated genes contain it less frequently (once in 119 bp) (Zürcher et al., 2013).

	sequence length	total hits	density: hits per N bp	distance (>30)	distance (6-30)	(6-30), tandem	(6-30), inverted
SPCH locus with 2.5 Kb upstream region	4912	52	94.46153846	38	7	3	3
MUTE locus with 1.8 Kb upstream region	3454	27	127.92592	19	8	2	6

Table S4. Description of experiments defining fraction of SLGC divisions, related to Figure 3, S3, Figure 4, and S4.

Detailed description of experiments indicating the total counts of divided and undivided SLGCs and the ratio of these counts and the number of cotyledons scored for each genotype and experiment.

	Genotype	Fraction of cells undergoing spacing divisions	%	Cotyledons scored
3D*	Col-0	189/306	61.8	13
	ckx346	442/619	71.4	19
3H	Col-0	128/243	52.7	7
	ahk3 cre1	91/203	44.8	7
	ahk2/+ ahk3 cre1	115/305	37.7	8
	ahk2 ahk3 cre1	28/266	10.5	7
3L	Col-0	145/289	50.2	7
	arr16 arr17 #1	200/303	66.0	7
	arr16 arr17 #2	187/287	65.2	7
	arr16 arr17 #3	187/292	64.0	7
3N	Col-0	264/465	56.8	18
	ARR16-OE	60/275	21.8	16
S3D*	Col-0	189/306	61.8	13
	ipt1357	149/323	46.1	17
	ipt1345678	132/379	34.8	15
S3H	Col-0	302/574	52.6	14
	arr16 #1	243/413	58.8	12
	arr16 #2	447/816	54.8	17
	arr16 #3	322/591	54.5	14
	arr16 arr17 #1	330/511	64.6	12
	arr16 arr17 #2	474/715	66.3	14
	arr16 arr17 #3	433/654	66.2	15
4H	Col-0	305/541	56.4	32
	cle9 cle10	606/819	74.0	32
	cle9 cle10; ARR16-OE	136/345	39.4	19
4K	Col-0	255/424	60.1	12
	arr16 arr17	298/428	69.6	13
	cle9 cle10	519/709	73.2	15
	arr16 arr17 cle9 cle10	421/581	72.5	15
S4F	Col-0	496/836	59.3	22
	cle9 #1	297/474	62.7	13
	cle9 #2	276/433	63.7	15
	cle9 cle10 #1	519/709	73.2	15
	cle9 cle10 #2	447/641	69.7	14
	cle9 cle10 #3	621/875	71.0	14
	cle9 cle10 #4	218/298	73.2	7

* The same Col-0 samples (grow together with *ckx* and *ipt* mutants) were used as controls in 3D and S3D

Table S5. List of primers used in this study, related to STAR methods.

Genotyping primers		
ckx4-1	ckx4-1 LP	GGTCACGGTGATTCGTAGATG
	ckx4-1 RP	AAACTCACACGCCATAACCAG
ckx3-2	ckx3-2 LP	ATACATGTGTCTGCATGCGTG
	ckx3-2 RP	TAGACCAACCACCAATTTTGC
ckx6-2	ckx6-2 LP	TCTACAGTGTGGATTCCCCTG
	ckx6-2 RP	CCTTTCGATCGGAGTTTTACC
ckx5-1	ckx5-1 LP	CGAGCTTCAAAGTGTTTACGG
	ckx5-1 RP	TCCCCACAAGTGTAACTTTGC
arr16	ARR16 UTR F	TACCATCACCAGAGATTCAC
	gARR16 R	GCTTCTGCAGTTCATGAGATGA
arr17	ARR17 pro F	GTCTGACTCATTTATTCTCCTCC
	gARR17 R	GCTTCTGCAATTTAAAAGATGG
cle9	CLE 9 seq F	CAAGCGTGGAAATAGCTTAGTTAC
	CLE 9 seq R	CGGGTTGGTATTTATGTCAATGAAAAC
cle10	CLE10 seq F	GATGCAGTAATAACAAGGAAGTGAAG
	CLE10 seq R	GGAAATTGTAACTTTCCGATAAAG
qRT-PCR primers		
ACTIN	ACTIN qPCR F	CAAGGCCGAGTATGATGAGG
	ACTIN qPCR R	GAAACGCAGACGTAAGTAAAAAC
ARR16	ARR16 qPCR F	TGCAAAGTGACAACAGCAGA
	ARR16 qPCR R	CCAGGCATACAGTAATCGGT
SPCH	SPCH qPCR F	GCTGCTCTTGAAGATTTGGCTC
	SPCH qPCR R	GCACTCAATTCCAATCTTGATGGTG
MUTE	MUTE qPCR F	CAAAAGGGGAGATCAAGCTTCG
	MUTE qPCR R	GGTCTTTCGACGTTTCTTGGAC
Primers used for DNA manipulation		
CKX4 pro	CKX4 pro F	CCCCACGCGTTGTTGCTAAGCTCTTCTTCTTCTTCTTCGA
	CKX4 pro R	CCCCACGCGTTGTTGCTAAGCTCTTCTTCTTCTTCTTCGA
ARR16 pro	ARR16 pro F	AATATGAATTCTAAACTCAGTTGATACTTC
	ARR16 pro R	AATATGAATTCGATCTCCTTTGTTCCTTAAA
ARR17 pro	ARR17 pro F	AATATGAATTCGATCTCCTTTGTTCCTTAAA
	ARR17 pro R	CTCCTACGATTACAGAGAGAT
ARR18 pro	ARR18 pro F	AATATGAATTCGATCTCCTTTGTTCCTTAAA
	ARR18 pro R	TCCTACAGGAAACTTGTCATGC
ARR22 pro	ARR22 pro F	GTGATTTCTGTCAACTAAATG
	ARR22 pro R	CTTCGATTTCTTTTCTCTCA
CLE9 pro	CLE9 pro F	ATCAGAACCTGAGAACTATAG
	CLE9 pro R	TGTTTTGGTTTCCAAGAGAG
CLE10 pro	CLE10 pro F	GATTCAACAAAACCGGTACAACG
	CLE10 pro R	CGTTGTGGAGAGAGAGAGAGAG
TCSn1 pro	TCSn1 pro F	CCCTCGAGCCCGGGTCCTCTCCAAATGAAATGAACTTCCTTATATA
	TCSn1 pro R	CCCACGCGTAAGCTTGACTAGTCAAAGATCTTTAA
pENTR CKI1	pENTR CKI1 F	CACCATGATGGTGAAAGTTACAAAGC
	pENTR CKI1 R	CTAGTGACGTTTGCTTTCGA
pENTR CKX3	pENTR CKX3 F	CACCAAAAAAATGGCGAGTTATAATCTTCGTT
	pENTR CKX3 R	CTAACTCGAGTTTATTTTTTGAAAT
pENTR IPT7	pENTR IPT7 F	CACCGCCACCATGAAGTTCTCAATCTCATCACTGAAGC
	pENTR IPT7 R	TCATATCATATTGTGGGCTCTACTT
pENTR ARR16	pENTR ARR16 F	CACCATGAACAGTTCAGGAGGTT
	pENTR ARR16 R	GCTTCTGCAGTTCATGAGATGA
pENTR ARR17	pENTR ARR17 F	CACCATGAATAAGGGCTGTGGAAG
	pENTR ARR17 R	GCTTCTGCAATTTAAAAGATGG
pENTR ARR18	pENTR ARR18 F	CACCATGAGGGTTCTTGCTGTGGA
	pENTR ARR18 R	AGGTGGAGGAAATGAATCAAAGCC
pENTR SPCH	pENTR SPCH F	CACCATGCAGGAGATAATACCGG
	pENTR SPCH R	GCAGAATGTTTGCTGAATTTGTTGAGCC

Table S6. Description of all transgenic lines created and analyzed, related to STAR methods.

Transgenic lines created for this study	Nro of independent lines analyzed
pCKX4-2xΩYpet-N7	*9*
pARR16-gARR16-CFP	9
pARR22-YFP	*8*
pARR18-gARR18-YFP	9
pARR17-gARR17-YFP	5
pML1-CKX3	*13*
pML1-IPT7	*9*
pBASL-CKX3	*9*
pBASL-IPT7	*14*
pBASL-CKI1	*25*
pMUTE-CKX3	*8*
pMUTE-IPT7	*14*
pMUTE-CKI1	*12*
pTMM-gARR16-YFP	*13*
pARR17-GUS	10
pCLE9-YFP	12
pCLE10-YFP	*8*
pTCSn1-2xΩYpet-N7 in *cle9 cle10 #1*	*11*
pTMM-ARR16-YFP in *cle9 cle10 #1*	*10*
pBASL-CKI1 in *mute-/+*	*15*
pSPCH-SPCH-YFP in arr16 arr17 #1	*5*